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Q-One Biotech Limited
Todd Campus
West of Scotland Science Park
Glasgow
G20 0XA.

Patents ADP number *(if you know it)*

6602470021

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4. Title of the invention

"Porcine Retrovirus"

5. Name of your agent *(if you have one)*

Cruikshank & Fairweather

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Porcine Retrovirus

The present invention relates to porcine retrovirus (PoEV) fragments, in particular polynucleotide fragments encoding at least one porcine retrovirus expression product, a recombinant vector comprising at least one polynucleotide fragment, use of PoEV polynucleotide fragments in the detection of native porcine retrovirus, a host cell containing at least one PoEV polynucleotide fragment or a recombinant vector comprising at least one PoEV polynucleotide fragment, PoEV polypeptides, antibodies immuno-reactive with PoEV polypeptides and pharmaceutical compositions comprising recombinant PoEV polypeptides for use as prophylactic and/or therapeutic agents.

Porcine retrovirus (PoEV) is an endogenous (genetically acquired) retrovirus isolated from pigs and expressed in cell lines derived from porcine material. There are no known pathogenic effects associated with the virus per se in its natural host although the virus appears to be associated with lymphomas in pigs and related viruses are associated with leukaemias and lymphomas in other species. The virus has been reported to infect cells from a variety of non-porcine origins and is, therefore, designated as a xenotropic, amphotropic or polytrophic virus (Lieber MM, Sherr CJ. Benveniste RE and Todaro GJ. 1975; Strandstrom H, Verjalainen P, Moening V, Hunsmann G, Schwarz H, and Schafer W. 1974; Todaro GJ, Benveniste RE, Lieber MM and Sherr CJ. 1974). Such viruses have the potential to infect humans and may have a pathogenic effect thereon. The use

of pig tissues and cells in xenotransplants and treatment of diabetes in humans gives rise to concerns about the virological safety of such material. Information on the properties of PoEV and the development of diagnostic reagents, molecular engineering tools and potential vaccine materials is of paramount importance for example in xenotransplantation technology and the like.

It is an object of the present invention to obviate and/or mitigate against at least some of the above disadvantages.

The present invention provides an isolated PoEV polynucleotide fragment which is capable of encoding at least one PoEV expression product. Preferably, the polynucleotide fragment encodes the polymerase gene (*pol*) and/or envelope (*env*) gene of PoEV. A further expression product can be the polymerase (POL) and/or envelope (ENV) polypeptides of PoEV. Thus, the invention further provides a recombinant PoEV polymerase and/or envelope polypeptide.

"Polynucleotide fragment" as used herein refers to a chain of nucleotides such as deoxyribose nucleic acid (DNA) and transcription products thereof, such as RNA. Naturally, the skilled addressee will appreciate the whole naturally occurring PoEV genome is not included in the definition of polynucleotide fragment.

Generally the polynucleotide fragment will be in isolated form substantially free of biological material with which the whole genome is normally associated <u>in vivo</u>. Thus, "polynucleotide fragment" includes double and single stranded DNA, RNA and polynucleotide sequences derived therefrom, for example, subsequences of said fragment and which are of any

desirable length.

In general, the term "expression product" refers to both transcription and translation products of said polynucleotide fragments. When the expression product is a "polypeptide" (i.e. a chain or sequence of amino acids displaying a biological activity substantially similar to the biological activity of PoEV polymerase and/or envelope protein), it does not refer to a specific length of the product as such. Thus, the skilled addressee will appreciate that "polypeptide" encompasses inter alia peptides, polypeptides and proteins of PoEV. The polypeptide if required, can be modified in vivo and in vitro, for example by glycosylation, amidation, carboxylation, phosphorylation and/or part translational cleavage.

A polynucleotide fragment of approximately 3.2 kilobases (kb), comprising a portion of the PoEV genome, and derived from retrovirus particles released from a reverse transcriptase-positive porcine kidney cell line PK-15, has been molecularly cloned into a plasmid vector. This was achieved by synthesising cDNAs of PoEV RNA genomes which were recovered from porcine kidney cells expressing the endogenous virus. The cDNA was cloned into a plasmid vector and the DNA sequence of the isolated PoEV DNA fragment determined (see Figure 1). The DNA fragment of Figure 1 was shown to encode two open reading frames (ORFs) of 924 and 226 amino acids respectively. A comparison of the amino acid sequence against previously sequenced retroviruses from other species indicated that novel retrovirus cDNA had been cloned. Sequence analysis using the Lasergene software from DNASTAR Inc. showed that homologies were observed between the

cloned PoEV DNA and the majority of retroviruses and that the closest homologies were to gibbon leukaemia virus (GaLV) in the polymerase (*pol*) and (*env*) regions of the pro-virus. The large open reading frame ORF of Figure 1 (nucleotide 2-2773) is predicted to encode the PoEV polymerase polypeptide and the smaller ORF (nucleotide 2622-3275) is predicted to encode the PoEV envelope polypeptide.

The skilled addressee will appreciate that it is possible to genetically manipulate the polynucleotide fragment or derivatives thereof, for example to clone the gene by recombinant DNA techniques generally known in the art and to express the polypeptides encoded thereby in vitro and/or in vivo. DNA fragments having the polynucleotide sequence depicted in Figure 1, or derivatives thereof, may be used as a diagnostic tool or as a reagent for detecting PoEV in pig lines, or as a vaccine.

The polynucleotide fragments of the present invention may be used to examine the expression of the PoEV virus in donor animals and tissues derived from the donor animals suitable for xenotransplantation. In addition, the recipients of pig organs can be examined for the presence and expression of PoEV virus.

Thus, the present invention further provides an isolated PoEV polynucleotide fragment capable of hybridising to a PoEV polynucleotide sequence. In this manner, the present invention provides probes for use in ex vivo and/or in situ PoEV virus detection and expression studies.

"Capable of hybridising" is taken to mean that under conditions of high stringency (i.e. conditions which allow duplex formation only between substantially identical complementary

polynucleotide fragments) said polynucleotide fragment will hybridise to complementary PoEV polynucleotide sequence in preference to polynucleotide sequences of other virus, animal species or otherwise. In a preferment the PoEV fragment binds to native PoEV.

The skilled addressee will appreciate how polynucleotide fragments may be designed and used as probes in polymerase chain reaction (PCR) experiments or Southern analysis for detecting the presence or otherwise of PoEV polynucleotide in pigs or in tissue samples taken from potential transplant organs such as liver, kidney and heart. Furthermore the polynucleotide fragments of the present invention can be used to analyze the genetic organisation of endogenous PoEV located in the animal cell genome in pigs thus permitting the screening of herds of pigs for provirus loci (e.g. non-expressed provirus loci). Such a screening method would facilitate, for example, screening in a population of animals which are bred to lack expressed provirus loci.

Reagents may also be developed from said polynucleotide fragments as aids in breeding programmes and to generate pigs substantially free from native PoEV infection. *pol* and/or *env* gene "knockouts" may be constructed to allow development of breeding programmes in pigs whereby native PoEV infection is substantially prevented or reduced. For example the nucleotide sequence of PoEV can be manipulated e.g. by deletion of a coding sequence <u>in vitro</u> and the resulting construct used to replace to the natural PoEV sequence by recombination. Thus, the provirual genome can be rendered inactive in the porcine cells. The

knockouts can be manipulated into embryos and/or stem cells and if required manipulated nuclei can be transferred from target cells to germ cells using micromanipulation techniques well known in the art. The invention also extends to animals derived from such germ cells.

The generation of reagents to suppress the expression of native PoEV loci in pigs, such as, by generation of antisense mRNAs, ribozymes or other antiviral reagents may also be developed.

The polynucleotide fragment can be molecularly cloned into a prokaryotic or eukaryotic expression vector using standard techniques and administered to a host. The expression vector is taken up by cells and the polynucleotide fragment of interest expressed, producing protein. Presentation of the protein on cell surface stimulates the host immune system to produce antibodies immunoreactive with said protein as part of a defence mechanism. Thus, expressed protein may be used as a vaccine.

Inactivated vaccines can be produced from PoEV's or cells releasing PoEV. Such infected cells may be generated by natural infection or by transfection of a proviral [Please define] clone of PoEV. It will be understood that a proviral clone is a molecular clone encoding on at least one antigenic polypeptide of PoEV. After harvesting the virus and/or the infected cells, viruses or infected cells present can be inactivated for example, with formaldehyde, gluteraldehyde, acetylethylenimine or other suitable agent or process to generate an inactivated vaccine using methods commonly employed in the art. (CVMP Working Party on Immunological Veterinary Medicinal Products (1993). General

requirements for the production and control of inactivated mammalian bacterial and viral vaccines for veterinary use).

Proviral clones of PoEV can be engineered to develop single cycle or replication defective viral vectors suitable for vaccination using techniques. Such viral vectors known in the art (e.g. MuLV Murine Leukaemia Retrovirus, Adenovirus and Herpesviruses (Anderson WF. (1992). Human Gene Therapy. *Science* **256**, 808-813) may have one or more genes essential for replication deleted, with the missing gene function expressed constitutively or conditionally from a further, different construct which is integrated into the chromosomal DNA of a complementing cell line to the proviral PoEV clone. PoEV virions released from the cell line may infect secondary target cells in the vaccinee but not produce further infectious virus particles. For instance, the polynucleotide sequence encoding the reverse transcriptase domain of *pol* can be deleted from the proviral PoEV clone and the reverse transcriptase domain of *pol* integrated into the complementing cell line.

It will be understood that the polynucleotide and polypeptides encompassed by the present invention could be used in therapy, or methods of treatment.

The cloning and expression of a recombinant PoEV polynucleotide fragment also facilitates in producing anti-PoEV antibodies (particularly monoclonal antibodies) and evaluation of <u>in vitro</u> and <u>in vivo</u> biological activity of recombinant PoEV polymerase and/or envelope polypeptides. The antibodies may be employed in diagnostic tests for native PoEV virus.

It will be understood that for the particular PoEV

polypeptides embraced herein, natural variations can exist between individuals or between members of the family Suidae (i.e. the pig family). These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. All such derivatives showing active polymerase and/or envelope polypeptide physiological activity are included within the scope of the invention. For example, for the purpose of the present invention conservative replacements may be made between amino acids within the following groups:

(I) Alanine, serine, threonine;

(II) Glutamic acid and aspartic acid;

(III) Arginine and leucine;

(IV) Asparagine and glutamine;

(V) Isoleucine, leucine and valine;

(VI) Phenylalanine, tyrosine and tryptophan

Moreover, recombinant DNA technology may be used to prepare nucleic acid sequences encoding the various derivatives outlined above.

As is well known in the art, the degeneracy of the genetic code permits substitution of bases in a codon resulting in a different codon which is still capable of coding for the same amino acid, e.g. the codon for amino acid glutamic acid is both GAT and GAA. Consequently, it is clear that for the expression of polypeptides with the amino acid sequences shown in Figure 1 or fragments thereof, use can be made of a derivative nucleic acid sequence with such an alternative codon composition different from the nucleic acid sequence shown in said Figure 1.

Furthermore, fragments derived from the PoEV polymerase and/or envelope polypeptides as depicted in Figure 1, which still display PoEV polymerase and/or envelope polypeptide properties, or fragments derived from the nucleic acid sequence encoding the polymerase and/or envelope polypeptides or derived from the nucleotide sequence depicted in Figure 1 encoding fragments of said polymerase and/or envelope polypeptides are also included of the present invention. Naturally, the skilled addressee will appreciate within the ambit that the said fragments should substantially retain the physiological properties of the *pol* and/or *env* polypeptides.

The PoEV polynucleotide fragment of the present invention is preferably linked to regulatory control sequences. Such control sequences may comprise promoters, operators, inducers, enhancers, ribosome binding sites, terminators etc. Suitable control sequences for a given host may be selected by those of ordinary skill in the art.

A polynucleotide fragment according to the present invention can be ligated to various expression controlling sequences, resulting in a so called recombinant nucleic acid molecule. Thus, the present invention also includes an expression vector containing an expressible PoEV nucleic acid molecule. The recombinant PoEV nucleic acid molecule can then be used for the transformation of a suitable host. Such hybrid molecules are preferably derived from, for example, plasmids or from nucleic acid sequences present in bacteriophages or viruses and are termed vector molecules.

Specific vectors which can be used to clone nucleic acid

sequences according to the invention are known in the art (e.g. Rodriguez, R.L. and Denhadt, D.T., Edit., Vectors: a survey of molecular cloning vectors and their uses, Butterworths, 1988).

The methods to be used for the construction of a recombinant nucleic acid molecule according to the invention are known to those of ordinary skill in the art and are inter alia set forth in Sambrook, et al. (Molecular Cloning: a laboratory manual Cold Spring Harbour Laboratory, 1989).

The present invention also relates to a transformed cell containing the PoEV polynucleotide fragment in an expressible form. "Transformation", as used herein, refers to the introduction of a heterologous polynucleotide fragment into a host cell. The method used may be any known in the art, for example, direct uptake, transfection or transduction (Current Protocols in Molecular Biology, 1995. John Wiley and Sons Inc). The heterologous polynucleotide fragment may be maintained through autonomous replication or alternatively, may be integrated into the host genome. The recombinant nucleic acid molecules preferably are provided with appropriate control sequences compatible with the designated host which can regulate the expression of the inserted polynucleotide fragment, e.g. tetracycline responsive promoter, thymidine kinase promoter, SV-40 promoter and the like.

Suitable hosts for the expression of recombinant nucleic acid molecules may be prokaryotic or eukaryotic in origin. Hosts suitable for the expression of recombinant nucleic acid molecules may be selected from bacteria, yeast, insect cells and mammalian cells.

Since the biological half life and the degree of glycosylation of recombinant PoEV polymerase and/or envelope polypeptides may be important for use in vivo, yeast and baculovirus systems, in which a greater degree of processing and glycosylation occur, are preferred. The yeast strain Pichia Pastoris exhibits potential for high level expression of recombinant proteins (Clare et al., 1991). The baculovirus system has been used successfully in the production of type 1 interferons (Smith et al., 1983).

Embodiments of the invention will now be described by way of example only.

Examples Section

Example 1

Preparation of viral RNA

RNA was prepared from the virus pellet using a Dynabeads (registered trade mark) mRNA Direct kit according to the manufacturer's protocols; A PoEV virus pellet was resuspended in 500µl of TNE (10mM Tris HCl pH8.0, 0.1M NaCl,1mM EDTA) and the virions disrupted by the addition of 1ml of lysis/binding buffer. Dynabeads Oligo$(dT)_{25}$ were conditioned according to the manufacturer's instructions and added to the virus disrupted solution. Viral RNA is allowed to bind to the Dynabead for 10 minutes before the supernatant is removed and the bound RNA was washed three times with washing buffer with LiDS (0.5ml) and twice with washing buffer alone. The RNA is final resuspended in 25 µl of elution solution. All procedures were performed at

ambient temperature. RNase contamination was avoided by the wearing of gloves, observation of sterile technique and treatment of solutions and non-disposable glass and plasticware with diethyl pyrocarbonate (DEPC). The RNA was resuspended in DEPC-treated sterile water.

Example 2

Synthesis of cDNA

cDNA was synthesised from the purified genomic RNA using Great Lengths ™ cDNA amplification reverse transcriptase reagents (Clonetech Laboratories Inc.) following the manufacturer's instructions. The RNA was primed with both oligo(dT) and random hexamers to maximise synthesis.

The Great Lengths cDNA synthesis protocol is based on a modified Gubler and Hoffman (1983) protocol for generating complementary DNA libraries and essentially consists of first-strand synthesis, second strand synthesis, adaptor ligation, and size fractionaction.

First strand synthesis: lock-docking primers anneal to the beginning of the poly-A tail of the RNA due to the presence of A, C or a residue at the 3'-end of the primer. This increases the efficiency of cDNA synthesis of eliminating unnecessary reverse transcription of long stretches of poly-A. In addition, the reverse transcriptase used is MMLV (RNase H^-) which gives consistently better yields than do wild-type MMLV or AMV reverse transcriptase.

Second strand synthesis: the ratio of DNA polymerase I for RNase H has been optomised to increase the efficiency of the second strand synthesis and to minimize priming by hair pin-loop formation. Following secoond-strand synthesis, the * cDNA is treated with T4 DNA polymerase to create blunt ends.

Adaptor ligation: the cDNA is ligated to a specially designed adaptor that has a pre-existing EcoRI "sticky end". The use of this adaptor, instead of a linker, eliminates the need to methylate and the EcoRI - digest the cDNA, and thus leaves internal EcoRI, sites intact. The adaptor is 5'-phosphorylated at the blunt end to allow efficient ligation to the blunt-ended cDNA.

Size fractionation: the ds cDNA is phosphorylated at the EcoRI sites and size-fractionated to remove unligated adaptors and unincorporated nucleotides. The resulting cDNA is ready for cloning into a suitable EcoRI-digested vector.

Example 3

Molecular cloning of cDNA

The size fractionated fragment was ligated with EcoR I- digested pZErO™ -1 plasmid vector DNA (Invitrogen Corporation, San Diego, U.S.). The ligation mix was used to transform competent TOP10F'cells and these were plated onto L-Agar containing zeocin following the manufacturer's instructions (Zero Background™ cloning kit - Invitrogen). Several of the resulting zeocin resistant colonies were amplified in L-Broth containing zeocin and the plasmid DNA was purified by alkaline lysis (Maniatis *et*

al., 1982).

The plasmid DNA was digested to completion with the endonuclease EcoR I and the resulting DNA fragments were separated by electrophoresis through an 1.0% agarose gel (Maniatis *et al.*, 1982), in order to check that a fragment in the predicted size fractionated size range had been cloned. A clone identified as pPoEV was used in further experimentation.

Example 4

DNA sequence analysis.

pPoEV plasmid DNA was purified according to common techniques (Sambrook et al, 1989) and sequenced using an ABI automated sequencer. Overlapping sequencing primers from both strands of the molecular clone were used to determine the nucleotide sequence. Homologies were observed between pPoEV and the majority of retroviruses determined by using alogrithims from DNASTAR Inc. Lasergene software (DNASTAR). The homologies were closest with gibbon ape leukaemia virus (GaLV) in the polymerase (*pol*) regions of the pro-virus at 60.7%. The nucleotide sequence and major ORFs of the pPoEV insert are shown in Figure 1. The large ORF (nucleotide 1 - 2768) encodes polymerase polypeptide and the smaller ORF (nucleotide 2620 - end) encodes the envelope polypeptides.

Example 5

Purification of cellular DNA from cultured cells, tissues and blood.

Cultured cells

Cells were maintained in culture and approximately 5 x 10^7 cells were harvested for DNA preparation. The cells were pelleted by centrifugation resuspended in phosphate-buffered saline, re-centrifuged at 1000g for 2 minutes and the supernatant was discarded.

Porcine tissues

Porcine tissue samples were frozen in liquid nitrogen and powdered by grinding in a mortar or between metal foil. The samples were resuspended in 5ml of extraction buffer consisting of 0.1M EDTA (pH 8.0), 0.01MTris.Cl pH 8.0, 20μg/ml RNAse and 100μg/ml proteinase K (Maniatis *et al.*, 1982).

Porcine blood

A buffy coat was prepared from the blood samples. 20ml samples were centrifuged at 1000g for 15 minutes. The buffy coat was resuspended in buffer and the samples centrifuged at 1000g for 15 minutes. The process was repeated one further time. The sample was mixed with 5ml (3x volume) of extraction buffer (Maniatis *et al.*, 1982).

Purification

The samples (i.e. cultured cells, porcine tissue or porcine blood cells) in proteinase K-extraction buffer containing 20μg/ml RNAse

and 100µg/ml proteinase K were digested for approximately 24 hours at 37°C. The deproteinised DNA was extracted twice with phenol and twice with phenol chloroform and finally precipitated by ethanol in the presence of ammonium acetate. The DNA was recovered by centrifugation at 3000g for 30 minutes and the supernatant discarded (Maniatis *et al.*, 1982). The pellet was washed in 70% ethanol and allowed to air dry for approximately 1 hour. The DNA was allowed to re-dissolve in Tris EDTA (TE) buffer and the purity and concentration of the DNA was assessed by spectrophotometry (Maniatis *et al.*, 1982).

Example 6

Southern blot analysis of porcine tissue and cells

In order to demonstrate that the molecularly cloned DNA comprising the insert from PoEV was derived from the PK-15 cell line (American Type Culture Collection CCL33), the DNA was hybridised against cellular DNAs and its ability to detect proviral DNA was examined

DNA purified from pPoEV was radioactively labelled and used to probe a Southern blot of endonuclease digested DNAs derived from PK-15 cells .

The DNAs probed were as follows :

a) Copy number controls of pPoEV DNA linearized by digestion with EcoRI. One copy per haploid cell genome was estimated to be 6.84pg. The control was present at an estimated copy

number of 1, 5 and 10 copies.

b) PK-15 DNA.

c) Negative control HeLa (American Type Culture Collection CCL2) DNA derived from a human adenocarcinoma cell line harbouring human papillomavirus type 18 DNA.

d) Negative control SP20 (European Collection of Animal Cell Cultures 85072401) DNA derived from a murine myeloma cell line harbouring a xenotropic MuLV retrovirus.

A hybridisation signal was observed in only the PK-15 porcine DNA. No signal was detected in either the negative human or murine DNAs. The PK-15 DNA contained more than 10 copies per cell with an estimated copy number of 20. The sizes of the three major EcoRI- endonuclease digested DNA fragments were approximately 3.8kb, 1.8kb and 0.6kb. The sizes of relevant fragments detected in the recombinant pPoEV were comparable.

There are, as expected, a number of fragments common to the genomic DNA of PK-15 and pPoEV DNA and there is agreement between the patterns observed in both DNAs digested with XhoI, BamHI and HindIII. However, there are additional fragments obtained on digestion of pPoEV DNA by a number of endonucleases.

pPoEV sequences were also detected in swine testes (American Type Culture Collection CRL 1746) and primary porcine kidney cells

(Central Veterinary Laboratory batch C04495) but not in hamster CHOK1 (American Type Culture Collection CCL61) or murine NS0 myeloma cells (European Collection of Animal Cell Cultures 85110503).

In order to demonstrate that the molecularly cloned DNA comprising the insert from pPoEV could detect sequences in porcine cells and tissues in addition to PK-15 the pPoEV DNA was hybridised against cellular DNA from tissues derived from pigs and its ability to detect proviral DNA was examined (Maniatis et al., 1982).

The DNA purified from pPoEV was radioactively labelled and used to probe a Southern blot of endonuclease digested DNAs derived from pig organs including liver, kidney, heart and blood.

The DNAs probed were as follows :

a) Copy number controls of pPoEV DNA linearized by digestion with EcoRI. One copy per haploid cell genome was estimated to be 6.84pg. The control was present at an estimated copy number of 5,10, 20 and 50 copies.

b) DNA purified from the porcine tissues digested with EcoRI.

A hybridisation signal was observed in all the porcine DNAs.

The DNAs contained less than 5 copies per cell. There were

approximately eight distinct bands in each DNA. The sizes of the three major endonuclease digested DNA fragments were approximately 3.8kb, 1.8kb and 0.6kb.

Example 7

Polymerase Chain Reaction (PCR) Amplifications

Oligonucleotides were selected from the PoEV genome.

The upstream primer was 5'-GGA AGT GGA CTT CAC TGA G-3'.

The downstream primer was 5'-CTT TCC ACC CCG AAT CGG -3'.

The PCR was performed as described by Saiki *et al* (1987). One 1μl of 100ng/μl template DNA was added to a 49μl reaction mixture containing 200μM of dATP, dCTP, dGTP, dTTP, 30pmol of both primers from the pair described above, 1unit of DNA polymerase and 5μl of reaction buffer. The reaction buffer contained 200mM Tris-HCl pH 8.4, 500mM potassium chloride and 15mM magnesium chloride, ultrapure water. The solution was overlaid with two drops of mineral oil to prevent evaporation. Thirty five cycles of amplification were performed using a Perkin Elmer Cetus thermal cycler. Each cycle consisted of 1 minute. at 95^{0}C to denature the DNA, 1 minute. at 53°C to anneal the primers to the template and 1 minute. at 72^{0}C for primer extension. After the last cycle a further incubation for 10 minutes. at 72^{0}C was performed to allow extension of any partially completed product. On completion of the amplification, 10μl of the reaction mixture was rophoresed through a 5 per cent acrylamide gel. The DNA was

visualised by staining with ethidium bromide and exposure to ultraviolet light (320nm).

The PCR reaction amplified a sequence of approximately 787bp from pPoEV and from porcine cells as expected indicating that the assay detected the PoEV proviral DNA. There was no specific amplification of the expected sequence in cells of non-porcine origin and therefore, the PCR reaction and recombinant clone can be used as a specific and sensitive diagnostic tool for detection of PoEV.

Example 8

Production of PoEV polypeptide in *Escherichia coli*.

The open reading frame (ORF) encoding the *pol* peptide was isolated from the pPoEV clone and molecularly cloned into the plasmid pGEX-4T-1 (Pharmacia Ltd.) for expression.

Two ml cultures of *E. coli* transformed with various expression constructs were grown with shaking at 37°C to late log phase ($O.D._{600nm}$ of 0.6) and induced by the addition of IPTG to 0.1 mM. Induced cultures were then incubated for a further 2 hours after which the bacteria were collected by centrifugation. The bacterial pellet was lysed by boiling in SDS-PAGE sample buffer and the protein profile of the induced bacteria was analysed on a 12% acrylamide gel (Laemmli, 1970) followed by staining with coomassie brilliant blue dye.

References

Clare JJ, Rayment FB, Ballantine SP, Sreekrishna K and Romanos MA. (1991). High-level expression of tetanus toxin fragment C in *Pichis pastoris* strains containing multiple tandem integrations of the gene. *Biotechnology*, **9**, 455-460.

Derynck R, Singh A and Goeddel DV. (1983). Expression of the human interferon-γ in yeast. *Nucleic Acids Res.*, 11, 1819-1837.

DNASTAR. (1994).Lasergene Biocomputing Software for Windows. User's Guide.

Invitrogen. Version A. Zero Background™ Cloning Kit Catalog no K2500-01.

Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4N. *Nature*, **227**, 680-685.

Lieber MM, Sherr CJ, Benveniste RE and Todaro GJ. (1975). Biologic and immunologic properties of porcine type C viruses. *Virology* **66**, 616-619.

Maniatis T, Fritsch EF and Sambrook J. (1982). *Molecular Cloning : A Laboratory Manual*, Cold pring Harbour Laboratory, Cold Spring Harbour, NY.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB and Erlich HA. (1987). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**, 487-491.

Sambrook J, Fritsch EF, and Maniatis T. (1989). *Molecular Cloning: A laboratory Manual, 2nd ed.* Cold Spring Harbor Laboratory, Cold Spring Harbor New York.

Smith GE, Summers MD and Fraser MJ. (1983). Production of human beta interferon in insect cells infected with a baculovirus vector. *Mol. Cell. Biol.*, **3**, 2156-2165.

Strandstrom H, Verjalainen P, Moening V, Hunsmann G, Schwarz H. and Schafer W. (1974). C-type particles produced by a permanent cell line from a leukemic pig. 1 Origin and properties of host cells and some evidence for the occurrence of C-Type like particles *Virology* **57**, 175-178.

Todaro GJ, Benveniste RE, Lieber MM and Sherr CJ. (1974). Characterization of a type C virus released from the porcine cell line PK(15). *Virology* **58**, 65-74..

LONG ORF.

17 April 1996 16:26
TG8.MPD (1 > 3300) Site and Sequence
Enzymes: All 366 enzymes (No Filter)
Settings: Linear, Certain Sites Only, Standard Genetic Code

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  D  A  F  F  C  L  R  L  H  P  T  S  Q  P  L  F  A  F  E  W  R  D  P  G  T  G  R  T  G  Q
GCTCACCTGGACCCGACTGCCCCAAGGGTTCAAGAACTCCCCGACCATCTTTGACGAAGCCCTACACAGGGACCTGGCCAACTTCAGGAT                180
CGAGTGGACCTGGGCTGACGGGGTTCCCAAGTTCTTGAGGGGCTGGTAGAAACTGCTTCGGGATGTGTCCCTGGACCGGTTGAAGTCCTA
  L  T  W  T  R  L  P  Q  G  F  K  N  S  P  T  I  F  D  E  A  L  H  R  D  L  A  N  F  R  I
CCAACACCCTCAGGTGACCCTCCTCCAGTACGTGGATGACCTGCTTCTGGCGGGAGCCACCAAACAGGACTGCTTAGAAGGTACGAAGGC  270
GGTTGTGGGAGTCCACTGGGAGGAGGTCATGCACCTACTGGACGAAGACCGCCCTCGGTGGTTTGTCCTGACGAATCTTCCATGCTTCCG
  Q  H  P  Q  V  T  L  L  Q  Y  V  D  D  L  L  L  A  G  A  T  K  Q  D  C  L  E  G  T  K  A
ACTACTGCTGGAATTGTCTGACCTAGGCTACAGAGCCTCTGCTAAGAAGGCCCAGATTTGCAGGAGAGAGGTAACATACTTGGGGTACAG  360
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  L  L  L  E  L  S  D  L  G  Y  R  A  S  A  K  K  A  Q  I  C  R  R  E  V  T  Y  L  G  Y  S
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  L  R  G  G  Q  R  W  L  T  E  A  R  K  K  T  V  V  Q  I  P  A  P  T  T  A  K  Q  V  R  E
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  F  L  G  T  A  G  F  C  R  L  W  I  P  G  F  A  T  L  A  A  P  L  Y  P  L  T  K  E  K  G
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  G  F  S  W  A  P  E  H  Q  K  A  F  D  A  I  K  K  A  L  L  S  A  P  A  L  A  L  P  D  V
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  -  K  P  F  T  L  Y  V  D  E  R  K  G  V  A  R  G  V  L  T  Q  T  L  G  P  W  R  R  P  V
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  A  Y  L  S  K  K  L  D  P  V  A  S  G  W  P  V  C  L  K  A  I  A  A  V  A  I  L  V  K  D
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  T  N  A  R  M  T  H  Y  D  S  -  -  L  T  E  R  V  T  E  A  P  P  A  A  L  N  P  A  T  L
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Figure 1

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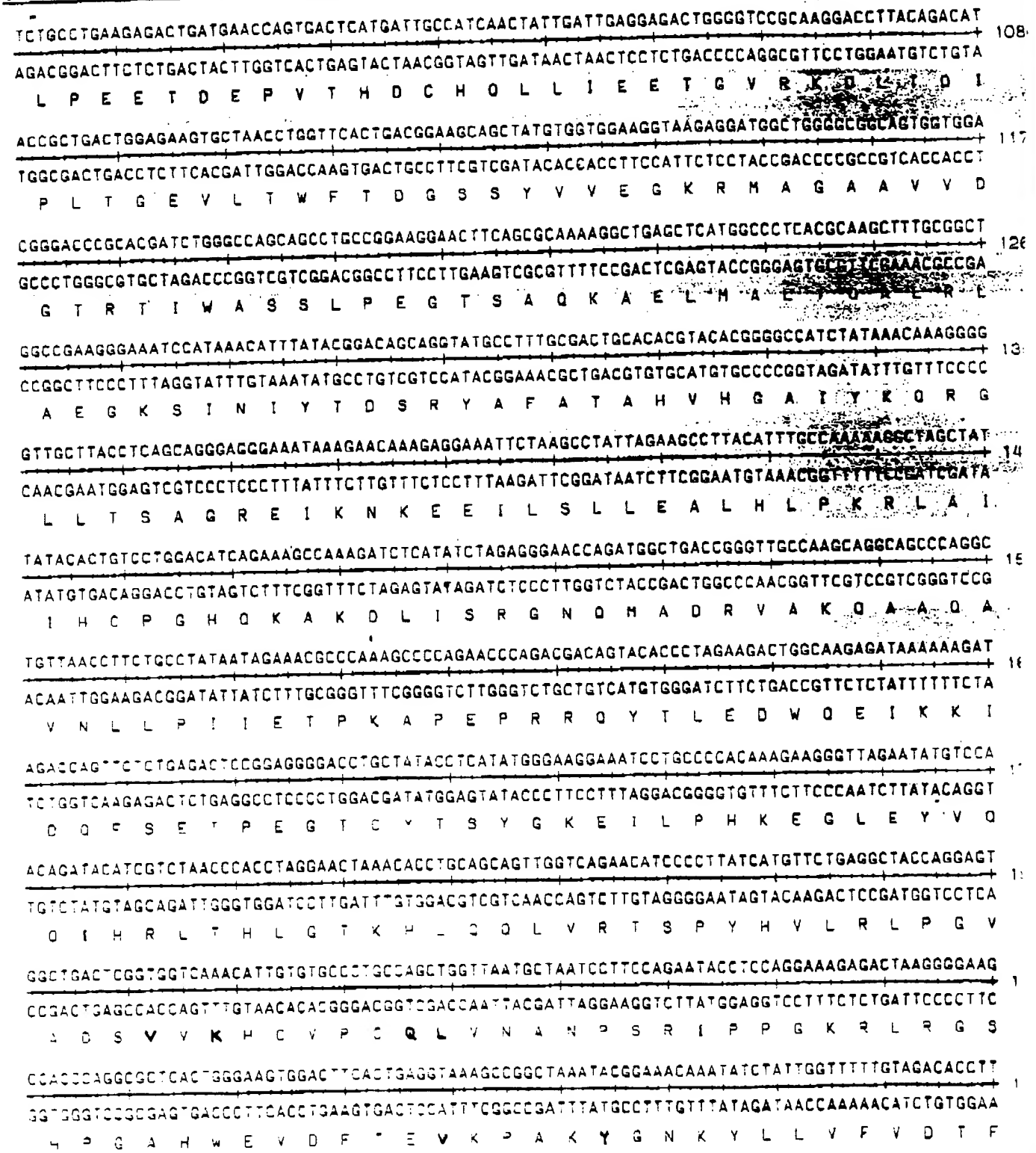
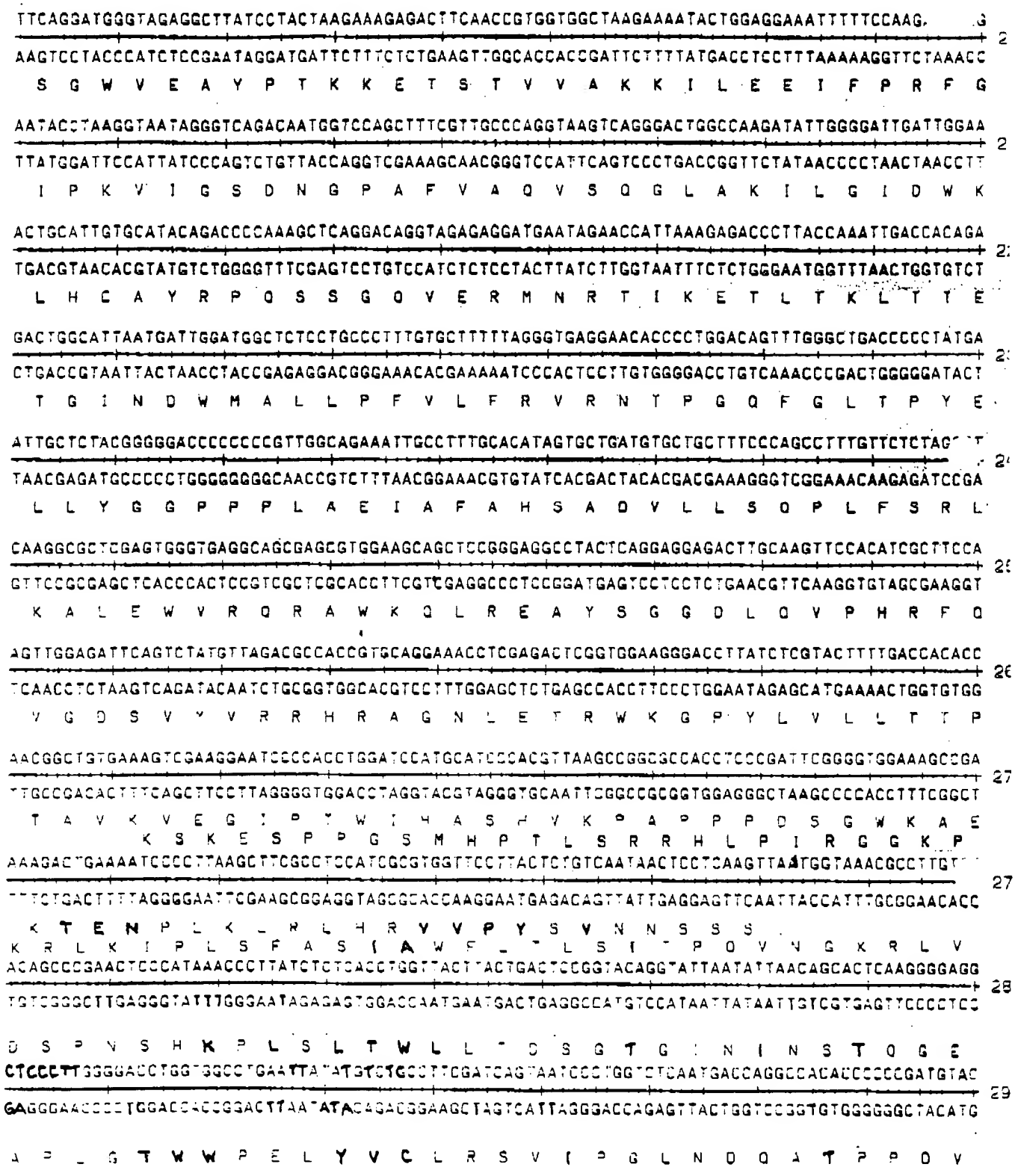
CTG8.MPD (1 > 3300) Site and Sequence





17 April 1996 16:26
CTG8.MPD (1 > 3300) Site and Sequence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                                                                                          3

  L  R  A  Y  G  F  Y  V  C  P  G  P  P  N  N  E  E  Y  C  G  N  P  Q  D  F  L  C  K  Q  W
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                                                                                          3

  S  C  I  T  S  N  D  G  N  W  K  W  P  V  S  Q  Q  D  R  V  S  Y  S  F  V  N  N  P  T  S
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TATTAGTTAAATTAATACCGGTACCCTCTACCTTTCTAACCGTTGTCGCCCATGTTTTTCTACATGCTTTATTCGTTTATTCGACAGTAA
                                                                                          3

  Y  N  Q  F  N  Y  G  H  G  R  W  K  D  W  Q  Q  R  V  Q  K  D  V  R  N  K  Q  I  S  C  H
CGTTAGACCTAGATTACTTAAAAATAAGTTTCACTAAAAAAAAAAAAAAAAAAAAAAAAAA
GCAATCTGGATCTAATGAATTTTTATTCAAAGTGATTTTTTTTTTTTTTTTTTTTTTTTTT
                                                                                       3300

  S  L  D  L  D  Y  L  K  I  S  F  T
```